REMARKS

Reconsideration of the above-identified application in view of the remarks following is respectfully requested.

Claims 1-11 are in this case. Claims 4-11 were Withdrawn under a restriction requirement as drawn to a non-elected invention. Claims 1-3 have been rejected. Claim 3 has now been Cancelled. Claims 1-2 have now been amended. New claims 12 and 13 have now been added.

35 U.S.C. §112 second paragraph rejections

The Examiner has rejected claims 1-3 under U.S.C. §112 second paragraph as being indefinite for failing to particularly point out and distinctly claim the subject matter which Applicant regards as the invention. The Examiner's rejections are respectfully traversed. Claim 1-3 have now been amended. New claim 12 has now been added.

The Examiner states that claim 1 is ambiguous and unclear in the recitation of "functional human β-2 microglobulin and functional human MHC class I".

Applicant wishes to point out that the term "functional" with respect to human β-2 microglobulin or human MHC class I is well-defined in the specification of the instant application.

Specifically, Applicant wishes to direct Examiner's attention to Page 21, lines 6-11 of the instant application which state as follows:

> *"As used herein the term "functional" when used in reference to the β-2 microglobulin and heavy chain polypeptides regions of a single chain MHC class I complex refers to any portion of each which is capable of contributing to the assembly of a functional single chain MHC class I complex (i.e., capable of binding and presenting to CTLs specific antigenic peptides when complexed)."* (Emphasis added)

Thus, the term "functional" refers to any biological characteristics (e.g., heterodimerization, peptide binding) of these polypeptides which ultimately enable the presentation and hence binding of soluble MHC class I complexes to CTLs.

For example, as shown in Example 1 of the Examples section of the instant application (pages 53-54, see also Figures 7a-b) compositions of the present invention containing the G9-209-2M peptide specifically activated the 209-specific CTL clone R6C12 but not the Mart-1 specific CTL clone JB2F4, indicating that the refolded scMHC-peptide complexes of the claimed invention are functional and specific.

Notwithstanding the above and in order to expedite prosecution of this case, Applicant has elected to amend claims 1 and 2 to better define the subject matter of the claimed invention.

Claims 1-2 have now been amended to recite the phrase "biologically functional", which clearly removes any lack of definition relating to the recitation of "functional" in the claim. Thus, Applicant is of the opinion that amended claims 1 and 2, thereby overcomes the Examiner's rejection.

Due to the vast amounts of information available with respect to the compositions of the present invention, and the guidelines and examples provided in the instant application, Applicant strongly believes that one of ordinary skill in the art privileged to the teachings of the present invention would be more than capable of selecting and synthesizing the compositions of the present invention which are capable of the described functional characteristics without having to resort to trial and error experimentation.

The Examiner states that claim 1 is ambiguous and unclear regarding the relationship of the "antigenic peptide" portion of the isolated chimeric polypeptide to the "functional human β-2 microglobulin" and "functional human MHC class I" portions of the chimeric polypeptide. The Examiner further states that claim 1 is ambiguous and unclear in the arrangement (*i.e.*, specific order or freeform) of the elements of the chimeric polypeptide.

The present invention relates to an isolated composition comprising a chimeric polypeptide composed of a recombinant (*i.e.*, expressed in host cells from a DNA construct) human β-2 microglobulin and a recombinant human MHC class I heavy chain, which are linked (to form a single polypeptide chain) in a manner which enables such a single polypeptide chain to bind the antigenic peptide (*i.e.*, in a functional arrangement).

It will be appreciated that various configurations of the composition of the present invention can be utilized as long as a functional assembly is obtained. For example, the isolated composition can be generated from a chimeric single chain polypeptide comprising the human β-2 microglobulin and the heavy chain of human MHC class I and an antigenic peptide which is either co-expressed from a separate DNA construct or separately synthesized.

Another example is a single chimeric polypeptide which comprises the human β-2 microglobulin, the heavy chain of human MHC class I and the antigenic peptide.

Support for such configurations can be found throughout the instant application. For example, Page 23, lines 12-16, describe a composition in which a recombinant chimeric polypeptide comprises the functional MHC heavy chain and the functional β-2 microglobulin with a linker peptide in between, while the antigenic peptide is either expressed from a second nucleic acid construct (Page 25, lines 12-16) or synthesized (as described on Page 38 lines 13-23, and Page 39 lines 1-2) and exogenously added following re-folding of the insoluble inclusion bodies comprising the single chain MHC class I complex (as described on Page 41, lines 9-22).

A chimeric polypeptide configuration can be generated using a nucleic acid construct in which the coding sequence of the human MHC class I heavy chain is translationally fused downstream to the coding sequence of human β-2 microglobulin (*i.e.*, the 3' end of the β-2 microglobulin is followed by the 5' end of the human MHC heavy chain, optionally using a linker peptide interposed therebetween, as described on Page 34, lines 3-9.

Alternatively, Page 23, lines 18-25 and Page 24, lines 1-20 teach a chimeric polypeptide in which the antigenic peptide is covalently linked upstream of the human β-2 microglobulin (*i.e.*, such that the 3'-end of the antigenic peptide is followed by the 5'-end of the β-2 microglobulin) directly or via a spacer (or linker) which is further conjugated (as described hereinabove) to the MHC class I heavy chain.

Not withstanding the above, and in order to expedite prosecution of the instant application, Applicant has elected to amend claim 1 to better define the claimed invention.

Thus, Currently Amended claim 1 recites:

> *"An isolated composition comprising a plurality of complexes each being composed of an antigenic peptide being capable of binding a human MHC class I and a chimeric polypeptide each comprising a biologically functional human β-2 microglobulin translationally fused to a biologically functional human MHC class I heavy chain and wherein said plurality of complexes are recognizable by a single specific CTL clone."* (Emphasis added)

Accordingly, new claim 12 which claims a chimeric polypeptide configuration which includes all three components has now been added.

Ample support for these claim amendments can be found throughout the instant application. As described on Page 34, lines 3-9, the human β-2 microglobulin and the MHC class I heavy chain can be expressed from a single nucleic acid construct in which the polynucleotide encoding the human MHC class I heavy chain is translationally fused (*i.e.*, covalently linked to form a single continuous open reading frame; Page 21, lines 15-20) downstream of the polynucleotide encoding the human β-2 microglobulin.

In view of the above arguments, Applicant believes to have overcome the 35 U.S.C. § 112, second paragraph, rejections.

35 U.S.C. § 103 rejections – Mottez in view of Lone

The Examiner rejected claims 1-3 under 35 U.S.C § 103(a) as being unpatentable over Mottez et al. (J. Exp. Med. [1995] 181:493-502) in view of Lone et al. (J. Immunotherapy [1998] 21(4):283-294). The Examiner's rejections are respectfully traversed. Claims 1-3 have now been amended. New claims 12 and 13 have now been added.

The Examiner states that Mottez et al. teach single chain constructs comprising a murine MHC class I heavy chain joined to β2-microglobulin with a covalently bound antigenic peptide, and that Lone teaches that the same techniques

were applied to human MHC class I heavy chain HLA-A2.1, which was joined via a 15-amino acid linker to human β2-microglobulin. The Examiner further states that according to Lone et al., the single chain MHC class I construct folded properly, was functional, and specifically bound HLA-A2 restricted peptides and induced peptide-specific cytotoxic T cells to proliferate and produce IL-2.

The present invention relates to an isolated homogeneous composition which comprises a plurality of complexes each including an antigenic peptide of a predetermined sequence which is capable of binding a human MHC class I and a recombinant functional human β-2 microglobulin translationally fused to a recombinant functional human MHC class I heavy chain. Such an isolated composition of well-defined components is recognizable by a single specific CTL clone and may serve as a valuable tool for *in vitro* studies of MHC-T-cell receptor (TCR) interactions and MHC-peptide complex structural analysis as well as for the inducement of specific T-cell responses and the *in vivo* modulation of immuno response for clinical purposes. It will be appreciated that for such applications, the components comprising the MHC-peptide complex of the present invention should be known and well-defined by their amino acid sequence.

In the interest of better distinguishing the claimed invention from the prior art, Applicant has elected to amend claim 1 as follows.

> *"An isolated composition comprising a plurality of complexes each being composed of an antigenic peptide being capable of binding a human MHC class I and a chimeric polypeptide each comprising a biologically functional human β-2 microglobulin translationally fused to a biologically functional human MHC class I heavy chain and wherein said plurality of complexes are recognizable by a single specific CTL clone."* (Emphasis added)

New claim 13 has been added.

> *"A bacterial inclusion body comprising a chimeric polypeptide which comprises a biologically functional human β-2 microglobulin translationally fused to a biologically functional human MHC class I heavy chain."*

Ample support for the added claim language can be found throughout the instant application, such as for example in page 1 lines 25-27.

> *"... which complexes are capable of presenting specific antigenic peptides restricted to class I MHC and recognizable by specific CTL clones or CD+8 T-cells."*

Support for expression and recovery of inclusion bodies expressing the complexes of the present invention can be found in the Examples section of the instant application (e.g., under the section "Expression, refolding and purification of scMHC-peptide complexes" of page 43 of the instant application).

Applicant wishes to point out that in sharp contrast to the homogeneous compositions of the present invention, Mottez and Lone describe a heterogeneous composition which comprises a mixture of endogenous and exogenously expressed MHC class I complex (*i.e.*, a β-2 microglobulin and MHC class I heavy chain) with an antigenic peptide.

Mottez et al., describe a eukaryotically expressed single polynucleotide encoding a mouse MHC molecule covalently bound to an antigenic peptide. Mottez et al. designed a DNA construct in which the oligonucleotide sequence encoding the antigenic peptide is ligated between the signal sequence and the mature sequence of the murine MHC Kd molecule, which are further ligated to the sequence encoding the murine β2 microglobulin. Following the expression of such a DNA construct in eukaryotic cells (*e.g.*, mouse L cells and CHO cells) Mottez et al., detected the antigenic peptide on the cell surface of the transfected cells and prepared soluble MHC complexes which were mistakenly assumed to include the recombinant antigenic peptide and the recombinant MHC I complex. In fact, since eukaryotic cells include endogenous MHC I complexes and a well-developed proteasome machinery, following the expression of such a DNA construct, which encodes the MHC I complex bound to the antigenic peptide, the recombinant MHC I complex is degraded by the endogenous proteasome machinery and the degraded antigenic peptide, which exhibits the inherent properties of a class I MHC peptide, further forms a complex with similar endogenous MHC I complex derived from the transfected cells (*i.e.*, resulting in a non-isolated, non-defined complex). Thus, contrary to the isolated

composition of the present invention, the compositions described by Mottez et al. will activate more than one CTL clone (such as when administered to a subject) and as such cannot be used for the in vitro studies of MHC-T-cell receptor (TCR) interactions and MHC-peptide complex or for clinical applications.

Similarly, Lone et al. generated a DNA construct that encodes a single chain polypeptide comprising the human β-2 microglobulin and human MHC-class I heavy chain, and expressed such a DNA construct in eukaryotic (*i.e.*, rat basophil) cells (RBL-2H3). As is mentioned hereinabove, in such eukaryotic cells the endogenously degraded peptides as well as the endogenously expressed HLA and β2-m polypeptides can form a complex with exogenously expressed HLA and/or β2-m and the exogenously added antigenic peptide. Thus, chimeric molecules generated using such eukaryotic cells as an expression system necessarily comprise a mixture of endogenous polypeptides and peptides of unknown sequences and exogenously expressed polypeptides (*i.e.*, via the expression vector) and exogenously added peptides. Thus, contrary to the isolated homogeneous composition of the present invention, the compositions described by Lone et al. will activate more than one CTL clone (such as when administered to a subject) and as such cannot be used for the in vitro studies of MHC-T-cell receptor (TCR) interactions and MHC-peptide complex or for clinical applications.

In addition, according to Lone et al., following the formation of the purified MHC class I polypeptide, the antigenic peptide was "loaded" (Lone et al., page 5, left column, lines 28-29) into the purified single chain MHC class I (SC-A2). However, it is well known that MHC class I polypeptides can not be correctly folded in the absence of a peptide [Fundamental Immunology, Forth edition; Lippincott-Raven publishers, pp. 273; Paul WE (Ed.)]. Therefore, the MHC class I polypeptide generated by Lone et al. was already bound to an endogenously expressed antigenic peptide. Thus, the "loading" step of the antigenic peptide performed by Lone et al. was actually an exchange reaction between the endogenous peptide and the exogenously provided peptide (e.g., NA17-A melanoma peptide). Such an exchange reaction involves stripping of the peptide, a reaction utilizing acid [Rotzschke O, et al., 1990, Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells, Nature. 348(6298): 252-4], which therefore results in destabilization

of the MHC-I heavy chain and β2-m complex [Rammensee HG, et al., 1993, MHC molecules as peptide receptors, Curr. Opin. Immunol. 5(1): 35-44; Urban RG, et al., 1997, The discovery and use of HLA-associated epitopes as drugs, Crit. Rev. Immunol. 17(5-6): 387-97]. Thus, due to the use of eukaryotic host cells and the further exchange reaction of the antigenic peptide, Lone et al. failed to obtain a recombinant MHC polypeptide in a complex with the exogenously added antigenic peptide. Thus, using the teachings of Mottez et al. and/or Lone et al. it is impossible to obtain the presently claimed isolated composition of a well-defined, synthetic or recombinant MHC-peptide complex of the present invention.

In contrast to prior art studies, the present invention teaches a soluble MHC molecule which is expressed in bacteria (e.g., *E. coli*) which lack the proteasome machinery. Such a bacterially expressed soluble MHC molecule is devoid of endogenous antigenic peptides and thus is available for binding peptides of interest.

This unique approach for expressing human MHC class I in prokaryotic cells enables isolation of functional MHC class I polypeptide since the synthesized protein is concentrated as insoluble inclusion bodies which can be recovered and refolded in the presence of the peptide to produce a functional and stable MHC I complexes, as a result, no peptide stripping and exchange is required.

Thus, in contrast to Examiner's statement, the isolated composition of the present invention which includes complexes of a biologically functional human MHC I complex, a biologically functional human β-2 microglobulin and an antigenic peptide capable of binding MHC I and are capable of activating a single CTL clone is novel and non-obvious with respect to the prior art cited.

In view of the above arguments, Applicant believes that claims 1-2 and newly added claims 12-13 are now in condition for allowance. Prompt notice of allowance is respectfully and earnestly solicited.

Respectfully submitted,

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Registration No. 40,338

Date: September 8, 2005

Enc.:

Two-month extension of time;

Four References:

1) Fundamental Immunology, Fourth Edition; Lippincott-Raven Publishers, pg. 273; Paul WE (Ed.)

2) Rotzschke, et al., 1990, Isolation And Analysis Of Naturally Processed Viral Peptides As Recognized By Cytotoxic T Cells, Nature. 348(6298): pg. 252-254.

3) Rammensee, et al., 1993, MHC Molecules As Peptide Receptors, Curr. Opin. Immunol. 5(1): pg. 35-44.

4) Urban, et al., 1997, The Discovery And Use Of HLA-Associated Epitopes As Drugs, Crit. Rev. Immunol. 17(5-6): pg. 387-397.

TABLE 6. *HLA disease associations*

Disease	Antigen	Race	Frequency[a]	
			Patients	Controls
Narcolepsy	HLA-DR2	C	1.0	0.22
		O	1.0	0.34
Ankylosing spondylitis	HLA-B27	C	0.89	0.09
		O	0.81	0.01
		N	0.58	0.04
Reiter's disease	HLA-B27	C	0.47	0.10
Insulin-dependent diabetes mellitus	HLA-B8	C	0.40	0.21
	HLA-B15	C	0.22	0.14
	HLA-DR3	C	0.52	0.22
	HLA-DR4	C	0.74	0.24
	HLA-DR2	C	0.04	0.29
	HLA-DRB1*0301	C	0.54	0.27
	HLA-DRB1*0401	C	0.59	0.25
	HLA-DQA1*0301	C	0.85	0.35
	HLA-DQB1*0302	C	0.81	0.23
Rheumatoid arthritis	HLA-DR4	C	0.68	0.25
		O	0.66	0.39
		N	0.44	0.10
Hodgkin's disease	HLA-A1	C	0.40	0.32
	HLA-DRB1*1104[b]	C	0.058	0.013
Hemochromatosis	HLA-A3	C	0.78	0.28
Psoriasis	HLA-Cw6	C	0.87	0.33
Celiac disease	HLA-DR3	C	0.79	0.26
Multiple sclerosis	HLA-DR2	C	0.59	0.26

C, Caucasian; O, Oriental; N, Black.
[a]The frequencies given are the total genotypic frequencies of all individuals with at least one copy of the designated allele. Both homozygous and heterozygous individuals are included.
[b]In this case, the frequencies are based on allele frequencies, not genotype frequencies.
Taken from *ref.* 113.

a more complete retrospective evaluation of the available data suggests that with the exception only of the *H-2K*^{k} gene, the spontaneous mutation rate for *H-2* genes was comparable to that for non-*H-2* genes (134). The characterization of these mutant animals, first based on peptide maps and amino acid sequences of the H-2 proteins (135–138) and later based on the nucleotide sequences of the cloned cDNAs or genes (101,102), provided some of the basic biochemical information on which later studies of structure and function and mechanism of gene evolution were based.

Expression of MHC Molecules

MHC molecules, synthesized in the ER and destined for cell surface expression, are controlled at many steps before their final disposition as receptors available for interaction with either T cells or NK cells. The MHC-I molecules should be viewed as trimers, consisting of the polymorphic heavy chain, the light chain, β2-microglobulin, and the assembled self peptide. Since there are numerous steps in the biosynthesis of the MHC-I molecule, regulatory controls can be exerted at almost every step. In addition, reflecting the continuous struggle between the immune system of the vertebrate organism, and rapidly adaptible infectious agents, a number of steps in biosynthesis and expression are inhibited by virus-encoded proteins.

The first level of control of MHC-I expression is genetic; that is, the genes for a particular chain must be present for the trimer to be expressed. This is of course most relevant for β2-microglobulin, which is the obligate light chain for the complex. Induced β2-m–defective animals (*β2m*$^{o/o}$) (139–141) lack normal levels of MHC-I expression, though for some molecules detectable amounts are present.

The next level of MHC-I expression control is transcriptional, and interferon-γ (IFN-γ) regulation is particularly important (142). For the most part, MHC-Ia molecules are ubiquitously expressed, and the basis of the more limited tissue-specific expression of MHC-Ib molecules is only beginning to be explored (143–145). Interest in the regulation of placental HLA-E and HLA-G expression is prompted by a potential role in the mother's tolerance of the fetus.

The rest of the MHC-I biosynthetic pathway is dependent on proper generation of cytosolic peptides by the proteasome and delivery to the ER by TAP, appropriate core glycosylation in the ER, transport through the Golgi, and arrival at the plasma membrane (146). A number of persistent viruses have evolved mechanisms for subverting this pathway of expression. The herpes simplex virus encodes a protein, ICP47, that blocks the activity of the peptide transporter TAP (147–149). Two proteins encoded by the human cytomegalovirus (HCMV), US2 and US11, cause rapid protein degradation of MHC-I molecules, and another HCMV protein, US18, which has sequence similarity to MHC-I molecules, may affect normal MHC-I function by limiting β2-m availability. The precise mechanism of US18 effects remains controversial. Several viruses, including murine cytomegalovirus (150), adenovirus 2

Isolation and analysis of naturally processed viral peptides as recognized by cytotoxic T cells

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VIRUS-infected cells can be eliminated by cytotoxic T lymphocytes (CTL), which recognize virus-derived peptides bound to major histocompatibility complex (MHC) class I molecules on the cell surface[1,2]. Until now, this notion has relied on overwhelming but indirect evidence, as the existence of naturally processed viral peptides has not been previously reported. Here we show that such peptides can be extracted from virus-infected cells by acid elution. Both the naturally processed $H\text{-}2\text{-}D^b$-restricted and $H\text{-}2\text{-}K^k$-restricted peptides from influenza nucleoprotein are smaller than the corresponding synthetic peptides, which have first been used to determine the respective CTL epitopes[3,4]. As with minor histocompatibility antigens[4], occurrence of viral peptides seems to be heavily dependent on MHC class I molecules, because infected $H\text{-}2^d$ cells do not contain the $H\text{-}2\text{-}D^b$-restricted peptide, and infected $H\text{-}2^b$ cells do not contain the $H\text{-}2\text{-}K^k$-restricted peptide. Our data provide direct experimental proof for the above notion on MHC-associated viral peptides on virus-infected cells.

Influenza virus strain A/PR/8/34 was used to infect EL4 ($H\text{-}2^b$) or P815-TR ($H\text{-}2^d$) mouse tumour cells, respectively. The virus-infected cells were subjected to an acid elution procedure that has been used to isolate naturally processed minor histocompatibility antigens[4,5]. Molecules with relative molecular masses less than 5,000 ($M_r < 5K$) contained in the resulting complex mixture were separated by reversed-phase HPLC (Fig. 1). Individual fractions were then tested for recognition by CTL specific for the dominant influenza virus epitopes, which are contained in amino-acid residues 365–380 from the nucleoprotein (NP; peptide NP365–380) for D^b-restricted CTL, and residues 147–158 from the same protein (NP147–158) for K^k-restricted CTL, respectively[1,3]. Material recognized by D^b-restricted CTL eluted at fraction 22 of the extract from infected, but not uninfected, EL4 cells, whereas none of the fractions was recognized by K^k-restricted CTL (Fig. 1*a–d*). By contrast, D^b-restricted peptides from influenza nucleoprotein are smaller than the corresponding synthetic peptides, which have first been used to determine the respective CTL epitopes[3,4]. As with minor histocompatibility antigens[4], occurrence of viral peptides seems to be heavily dependent on MHC class I molecules, because infected $H\text{-}2^d$ cells do not contain the $H\text{-}2\text{-}D^b$-restricted peptide, and infected $H\text{-}2^b$ cells do not contain the $H\text{-}2\text{-}K^k$-restricted peptide. Our data provide direct experimental proof for the above notion on MHC-associated viral peptides on virus-infected cells.

Influenza virus strain A/PR/8/34 was used to infect EL4 ($H\text{-}2^b$) or P815-TR ($H\text{-}2^d$) mouse tumour cells, respectively. The virus-infected cells were subjected to an acid elution procedure that has been used to isolate naturally processed minor histocompatibility antigens[4,5]. Molecules with relative molecular masses less than 5,000 ($M_r < 5K$) contained in the resulting complex mixture were separated by reversed-phase HPLC (Fig. 1). Individual fractions were then tested for recognition by CTL specific for the dominant influenza virus epitopes, which are contained in amino-acid residues 365–380 from the nucleoprotein (NP; peptide NP365–380) for D^b-restricted CTL, and residues 147–158 from the same protein (NP147–158) for K^k-restricted CTL, respectively[1,3]. Material recognized by D^b-restricted CTL eluted at fraction 22 of the extract from infected, but not uninfected, EL4 cells, whereas none of the fractions was recognized by K^k-restricted CTL (Fig. 1*a–d*). By contrast, D^b-restricted CTL which recognize virus-derived peptides bound to major histocompatibility complex (MHC) class I molecules on the cell surface[1,2]. Until now, this notion has relied on overwhelming but indirect evidence, as the existence of naturally processed viral peptides has not been previously reported. Here we show that such peptides can be extracted from virus-infected cells by acid elution. Both the naturally processed $H\text{-}2\text{-}D^b$-restricted and $H\text{-}2\text{-}K^k$-

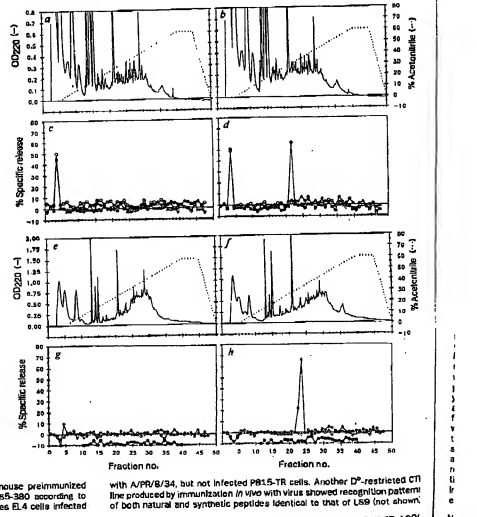

FIG. 1 Isolation of naturally processed viral CTL epitopes from virus-infected cells. EL 4 ($H\text{-}2^b$)(*a–d*) or P815-TR ($H\text{-}2^d$) (ref. 12) (*e–h*) tumour cells (8×10^8) were left untreated (left-hand panels) or were infected with 50,000 units of influenza A/PR/8/34 virus (right-hand panels). Peptides were isolated from cells by acid extraction[4,5] and separated by reversed-phase HPLC (*a, b, e, f*). Solid lines, absorption at 220 nm; dotted lines, percentage of acetonitrile in the gradient. *c, d* & *g, h*, individual HPLC fractions were tested for recognition by influenza virus-specific CTL lines LS9 (D^b-restricted, specific for an epitope on NP365–380) (●) or HASI (K^k-restricted, specific for an epitope on NP147–158) (◆) or with medium (○, ◇). Target cells were EL4 cells (○, ●) for LS9 and P815 ($H\text{-}2^d$) cells (◇, ◆) for HASI.

METHODS. Tumour cells were infected as described[6]. Cells were suspended in 0.1% trifluoroacetic acid (TFA), dounced, sonicated and centrifuged as described for spleen cells[5]. Material in the supernatant of $M_r > 5{,}000$ was removed by gel filtration (G25 Sepharose, Pharmacia). The remainder of the supernatant was separated on a reversed-phase HPLC column (Superpac Pep S, Pharmacia LKB) in 0.1% TFA using a gradient of increasing acetonitrile concentration. Flow rate, 1 ml min^{-1}; fraction size, 1 ml. Individual fractions were collected, dried, resuspended in PBS, incubated with ^{51}Cr-labelled tumour cells (either EL4 or P815) and tested for recognition by influenza-specific CTL in a standard ^{51}Cr release assay as described[2]. Spontaneous ^{51}Cr release of target cells ranged between 15 and 25%. Effector to target ratio ranged between 5:1 to 20:1. CTL line HASI was produced by stimulating spleen cells of a BALB/c mouse (preimmunized with 50 units of A/PR/8/34 virus) with 100 ng ml^{-1} of NP147–158 peptide in minimum essential alpha medium containing 10% FCS, β-mercaptoethanol, glutamine, and antibiotics at 37 °C, 5% CO_2, followed by weekly stimulation with irradiated (33 Gy) syngeneic spleen cells and peptide in medium supplemented with interleukin-2. This CTL line efficiently lyses A/PR/8/34-infected P815-TR, but not EL4 cells. The CTL line LS9 was produced by stimulating spleen cells of a (C57BL/6 × DBA/2)F1 mouse preimmunized with a synthetic lipopeptide vaccine[8] containing NP365–380 according to the protocol used to produce HASI. LS9 efficiently lyses EL4 cells infected with A/PR/8/34, but not infected P815-TR cells. Another D^b-restricted CTL line produced by immunization *in vivo* with virus showed recognition patterns of both natural and synthetic peptides identical to that of LS9 (not shown).

restricted CTL did not recognize any fraction from P815-TR extracts, whereas K^d-restricted CTL recognized fraction 24 from infected, but not from uninfected P815-TR cells (Fig. 1*e–h*). We conclude that naturally processed viral peptides exist, that they can be isolated from infected cells by acid elution, and that these peptides are involved in the MHC class I-restricted antigen-processing pathway, as suggested by their MHC dependency. The latter also excludes the possibility that the isolated peptides are artefacts produced during the extraction procedure. Because EL4 and P815-TR cells differ not only at MHC genes, the present data do not prove MHC dependency of processing ('processing' here means not only the cutting of proteins, but also the further fate of the degradation products). By use of MHC-recombinant and mutant mice, however, we have shown for minor histocompatibility antigens that the outcome of processing is dependent on MHC class I molecules[4].

For their molecular identification, the biochemical behaviour of naturally processed peptides was compared with that of the corresponding synthetic peptides. Figure 2*a*, *b* shows reversed-phase HPLC profiles of preparations of synthetic peptides NP147–158 (TYQRTRALVRTG (single-letter amino-acid code)) and NP365–380 (IASNENMETMESSTLE), respectively. Recognition of individual fractions by the respective CTL is shown in Fig. 2*c*, *d*. Not only the main products were recognized, but also some by-products of lower M_r (such by-products in small amounts are common to crude synthetic peptide preparations). This result is consistent with reports that CTL may recognize shorter peptides as well, and sometimes much better than the above peptides (for example, NP366–379)[1,5]. At higher dilution of fractions, D^b-restricted CTL failed to recognize the main peak, but still recognized other fractions, which contained small to undetectable (according to absorbance at 220 nm) amounts of peptide (Fig. 2*f*). Incidentally, both crude synthetic peptide preparations NP147–158 and NP365–380 contained other peptides of smaller size, which coeluted exactly with the respective natural peptide (Fig. 2*e*, *f*). Thus, the naturally processed virus peptides in both cases are different, most likely smaller, than the respective synthetic peptides reported[1,2] to contain CTL epitopes. Both natural peptides are recognized in a concentration-dependent and MHC class I-restricted manner (Fig. 3*a*, *d*). The shorter synthetic by-products coeluting with the natural peptides, again in both cases, are recognized much better than are the nominal synthetic peptides (Fig. 3*b*, *e*). A truncated peptide in the crude synthetic NP147–158 preparation coeluting with the natural peptide was determined by ion spray mass spectrometry to be TYQRTRALV. A subsequently synthesized peptide according to this sequence (NP147–155) coeluted with the natural peptide (not shown) and was indeed recognized (used as crude material) much better than NP147–158 (Fig. 3*c*), about as well as is TYQRTRALVTG (missing R at position 156) which is recognized 1,000-fold better than NP147–158 (ref. 6; Fig. 3*c*). Thus the natural K^d-restricted CTL epitope of influenza NP is likely to be TYQRTRALV. Under this assumption, comparison of the titration curves in Fig. 3*a*, *b*, *c* allows the calculation of the upper limit of peptide molecules extracted per infected cell to be 1,000. By similar analysis, the natural D^b-restricted peptide coeluted with ASNENMETM (NP366–374), which is recognized 1,000 times better than IASNENMETMESSTLE (manuscript in preparation). Thus, a previously identified optimal D^d-restricted NP peptide (NP366–379) happens to share its N-terminal amino acid residue with the natural peptide[1].

Together our experiments show that virus-infected cells produce small peptides from viral proteins which are recognized by MHC class I-restricted CTL, thereby directly confirming the overwhelming evidence provided by MHC crystallography and by experiments with synthetic peptides and truncated genes[1,2,7]. The data also indicate that the use of synthetic peptides to identify T-cell epitopes may be misleading, as very minor byproducts may be responsible for much of the biological effect.

The results in this and in a previous paper on naturally processed minor histocompatibility peptides[4] show two features of MHC class I-restricted antigen presentation. First, a cell produces and maintains exactly one peptide presented to a given

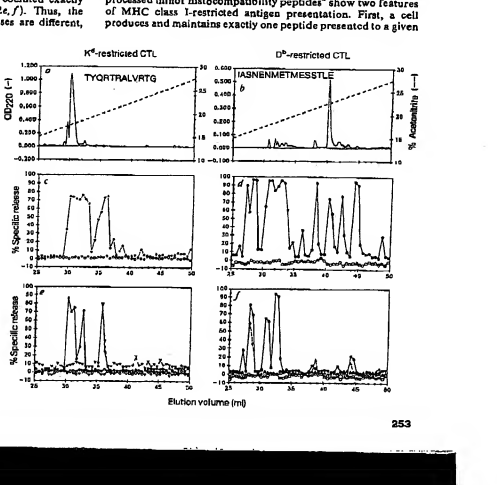

FIG. 2 Naturally processed influenza virus-derived CTL epitopes coelute with synthetic peptides. *a*, Reversed-phase HPLC (at higher resolution as in Fig. 1) elution profiles (solid lines) of synthetic peptide preparations according to NP147–158 (*a*) or NP365–380 (*b*). Dashed lines, percentage of acetonitrile in the gradient. *c*, Recognition of HPLC fractions (diluted 1:2,500) of the NP147–158 preparation by K^d-restricted CTL HASI (●) or without CTL (○) using P815 as target cells. *d*, Recognition of fractions of the synthetic NP365–380 preparation at same dilution by D^b-restricted CTL LS9 (●) or without CTL (○) using EL4 as target cells. *e*, Recognition of NP147–158 fractions (diluted 1:62,500) (●) and of rechromatographed natural K^d-restricted peptide (fraction 24 of Fig. 1*f*, *h*) (▼) by HASI CTL. *f*, Recognition of NP365–380 fractions (diluted 1:62,500) (●) and of rechromatographed natural D^b-restricted peptide (fraction 22 of Fig. 1*b*, *d*) (▲) by LS9 CTL.
METHODS. Peptides were synthesized as described[8]. Peptide preparations (400 μg of each) were chromatographed on the same reversed-phase HPLC column as in Fig. 1, using conditions yielding higher resolution (slower increase of acetonitrile concentration). Flow rate, 1 ml min^{-1}; fraction size, 0.5 ml. The main peaks in *e* and *f* were confirmed by ion-spray tandem-mass-spectrometry to be identical with the nominal peptide sequences, respectively. The fractions identified as naturally processed viral antigens in Fig. 1 were rechromatographed using exactly the same conditions as for the synthetic peptide preparations. Individual fractions were tested for CTL recognition as in Fig. 1.

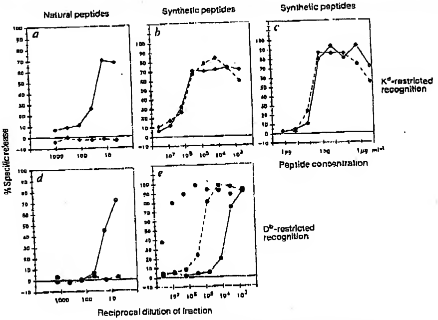

FIG. 3 Titration of natural and synthetic peptide fractions. *a*, Natural K^d-restricted NP peptide (fraction 24 of Fig. 1*f*, *h*) was tested in titrated concentrations for recognition by HASI CTL using P815 (—) or EL4 (- -) as target cells. No killing of EL4 indicates MHC class I-restricted recognition. *b*, Fractions of Fig. 2*a* representing the main synthetic peptide peak (TYQRTRALVRTG; 30.5–31.0 ml elution volume) (—) and the truncated peptide (TYQRTRALV according to tandem-mass-spectrometric analysis) coeluting with the natural peptide (35.5–36.0 ml) (- -) were tested in titrated dilution steps for recognition by HASI CTL. Note the difference between the peptide concentrations of the two samples, as evident from their absorption values in Fig. 2*a*. *c*, Crude batches of synthetic peptide preparations TYQRTRALV (- -) (synthesized on a fully automated simultaneous multiple peptide synthesizer; Zinsser Analytical model 350)[12] and TYQRTRALVTG (—) (missing R at position 156)[6,8] were tested in 10-fold dilution steps for recognition by HASI CTL. *d*, Natural D^b-restricted NP peptide (fraction 22 of Fig. 1*b*, *d*) was titrated into a CTL assay with LS9 CTL using EL 4 (—) or P815 (- -) as targets. *e*, Fractions of Fig. 2*b* representing the main synthetic peptide (IASNENMETMESSTLE; 40.0 to 40.5 ml elution volume) (—), the truncated synthetic peptide coeluting with the natural peptide (28.0–28.5 ml) (- -), and a second truncated, hyper-reactive peptide (32.0–32.5 ml) (· ·) were tested in 10-fold dilution steps for recognition by LS9 CTL.

CTL, as shown by elution of the natural peptide as a single sharp activity peak on reversed-phase HPLC profiles. Second, the processing of endogenous proteins seems to be dependent on MHC class I molecules, as discussed in ref. 4. A database containing many sequences of naturally processed peptides should allow insight into the specificity of the proteases involved, and further our knowledge about the rules of peptide-MHC interactions.

The acid elution method for isolation of naturally processed T-cell epitopes in combination with peptide chemistry including highly sensitive analytical tools such as HPLC and capillary-zone electrophoresis combined with ion-spray tandem-mass-spectrometry should provide straightforward experimental access to the peptide sequences of processed pathogen-derived antigens and has impact on synthetic vaccine design: the efficiency of synthetic peptide vaccines for CTL activation[8,9] could be improved further by using exactly the peptide produced by the infected cells. On the other hand, knowing exactly which peptide is presented naturally to a given T cell could help in the design of prophylactic or therapeutic measures for T cell-mediated autoimmune diseases[10]. □

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Three-dimensional structure of an idiotope–anti-idiotope complex

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Serologically detected antigenic determinants unique to an antibody or group of antibodies[1–3] are called idiotopes. The sum of idiotopes of an antibody constitute its idiotype[3]. Idiotypes have been intensively studied following a hypothesis for the self-regulation of the immune system through a network of idiotype–anti-idiotype interactions[4]. Furthermore, as antigen and anti-idiotypes can competitively bind to idiotype-positive, antigen-specific antibodies, anti-idiotypes may carry an 'internal image' of the external antigen (see refs 5–10 for reviews). Here we describe the structure of the complex between the monoclonal anti-lysozyme FabD1.3 and the anti-idiotopic FabE225 at 2.5 Å resolution. This complex defines a private idiotope consisting of 13 amino-acid residues, mainly from the complementarity-determining regions of D1.3. Seven of these residues make contacts with the antigen, indicating a significant overlap between idiotope and antigen-combining site. Idiotopic mimicry of the external antigen is not achieved at the molecular level in this example.

The crystal structure of the idiotope-anti-idiotope complex FabD1.3–FabE225 (ref. 11) shows the two Fabs roughly aligned along their major lengths, interacting largely through their complementarity-determining regions (CDRs) (Fig. 1*a*). Fourteen residues from the six CDRs of E225, together with a framework residue of V_L, contribute to interatomic contacts with the idiotope (Table 1). In turn, five CDRs and one V_L framework loop contribute 13 residues to the idiotope of D1.3 recognized by E225 (Table 2). FabE225 is centred on V_L of FabD1.3 such that nine residues of the idiotope are located on this domain

MHC molecules as peptide receptors

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The central unit for regulation of the specific immune system is a trimolecular complex made up of the T cell antigen receptor, the MHC molecule, and the MHC ligand. The third component is a peptide derived as a degradation product from a protein. During recent years there has been some progress in understanding the interaction between MHC molecules and their peptide ligands: MHC molecules are peptide receptors of peculiar specificity, being able to accommodate millions of different peptides provided they share some common features.

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Introduction

The function of MHC molecules is to collect peptides inside the cell and transport them to the cell surface, where they can be surveyed by T cells. The interaction between T-cell receptor (TCR), MHC molecule and the peptide presented by the MHC molecule is central to self/non self discrimination, that is, to the main theme of the immune system. The purpose of this review is to discuss the relationship between MHC molecules and their natural peptide ligands, with special emphasis on MHC class I molecules, because more is known about these than class II molecules.

Two classes of MHC molecules

Most of the human MHC genes are encoded on chromosome 6; those of the mouse are on chromosome 17 [1]. Each species has dozens of MHC loci, some of which contain pseudogenes. Some of the MHC genes are extremely polymorphic. Klein [2] distinguished two classes of MHC genes, class I and class II, a distinction that well anticipated the different physiological functions of the respective gene products. MHC class I molecules are made up of a heavy chain, comprising about 350 amino acids, and a light chain, comprising about 100 amino acids. The latter is also known as β2-microglobulin. There are about 50–50 MHC class I loci in the mouse and at least 17 in the human, including pseudogenes [1,3]. Most of this review will deal with only five of them, that is, HLA-A and -B, and H-2K, -D, and -L. MHC class II molecules are made up of an α-chain, comprising about 200 amino acids, and a β-chain of about the same size. There are about six α loci and 10 β loci for class II genes in the human and at least four α and three β loci in the mouse [1,3]; this review will only deal with HLA-DR, H-2A and H-2E molecules. Although MHC class I and class II molecules have many features in common, their function is quite different. Both classes will therefore be treated separately.

MHC class I molecules

Structure of class I molecules

The heavy chain consists of three extracellular domains, α1, α2, and α3, a transmembrane region [some non-classical class I molecules, however, are glycosyl phosphatidyl inositol (GPI)-anchored] and a cytoplasmic tail at the carboxyl terminus. The light chain, β2-microglobulin, is non-covalently attached to the heavy chain. Both α1 and α2 domains form a peptide accommodating groove, as revealed by X-ray crystallography of an A2 crystal by Bjorkman and colleagues in 1987 [4]. The groove is bordered by two α helices; the floor is a β-pleated sheet. The area of the groove is about 1 × 2.5 nm. The peptide appears to be an integral part of the protein complex, since empty class I molecules are thought to be rather unstable [5,6]. The peptide, which is generally between eight and 11 amino acids long, is tightly mounted in the groove in an extended configuration and certainly not as an α helix [9•,10•,11–15]. Both amino and carboxyl termini of the peptide are tightly bound via H-bonds by conserved residues. In addition, two side chains of residues at particular positions of the peptide, one at the carboxyl-terminus, the other elsewhere, depending on the MHC allele, are held by allele-specific pockets of the groove [9•,10•,11–18]. Monopeptidic crystals indicated that the spatial distance between the peptide's amino and carboxyl termini is constant, whereas the number of amino acids is not: the H-2K^b molecule, for example, can accommodate 8-mers in a stretched configuration, and also 9-mers with a kink in the middle [10•].

Abbreviations

HLA—human leukocyte-associated antigen; MHC—major histocompatibility complex; TCR—T-cell receptor; TFA—trifluoroacetic acid; VSV—vesicular stomatitis virus.

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The induction of kinks may be promoted by proline or glycine residues of the peptide [16]. For many class I molecules, such as H-2Kb, H-2Kd, and HLA-A2, the vast majority of natural peptide ligands are of uniform length (8 mers for H-2Kb and 9 mers for many others) [16]. For other class I molecules, such as HLA-A11 and HLA-A31, it seems that they can accommodate peptides comprising eight to 11 amino acid residues, all with a fixed carboxyl terminus (charged for HLA-A11) (K Falk, O Rötzschke, M Takiguchi *et al*, unpublished data). It seems, therefore, that the peptide backbone can bend more or less, to accomodate a different number of amino acids between the fixed MHC sites binding peptide amino and carboxyl termini as recently visualized by X-ray crystallography of Aw68 molecules and associated peptides [19].

Technical approaches for isolating natural MHC class I ligands

Peptides can be dissociated from class I molecules by treatment with acid (trifluoroacetic acid (TFA), or acetic acid) [16,20–23,24•,25–27]. Peptide loaded class I molecules need not be purified to undergo such treatment; treatment of whole cell lysate with TFA brings the peptides into solution, which can then be detected by the respective T cells [20]. This was the approach used for the first isolation of minor histocompatibility antigens as well as virus-derived peptides from cells [20–22]. Acid extraction of whole cells has the advantage of yielding not only MHC-bound peptides but also other peptides, for example potential intermediates of the processing pathway [21,22,24•,25]. The disadvantages are that one needs specific T cells to detect the peptides of interest, and that the resulting material is generally too complex for sequencing (although there is one exception [25]). If the MHC molecules are first purified and then acid extraction of peptides applied, the resulting peptide mixtures are much purer and they lend themselves more readily to further isolation and sequence analysis [16,23,28,29••,30•,31•,32].

Allele-specific peptide motifs

One of the first natural class I ligands to be identified was Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val from influenza nucleoprotein [22,33]. This peptide is presented by H-2Kd molecules of infected cells. The tyrosine residue of several synthetic peptides is known to be important for binding to H-2Kd, as well as an alanine or leucine about eight or nine residues from the tyrosine [34,35]. Comparison of Thr-Tyr-Gln-Arg-Thr-Arg-Ala-Leu-Val with the H-2Kd-binding synthetic peptides (all being longer than nine amino acids) suggested that all natural H-2Kd ligands might be nonamers with a tyrosine residue at position 2 and some aliphatic residue at position 9 [33]. We considered then that an obvious experiment would be to sequence all the H-2Kd ligands as a mixture to see whether the hypothesis was correct [16]. Indeed, pool sequencing of H-2Kd-eluted peptides gave a strong tyrosine signal at position 2, isoleucine and leucine at position 9, and lack of significant signals at position 10. Pool sequencing of peptides eluted from other MHC class I molecules indicated that each MHC allelic product has its own individual rules for peptide ligands, in most cases with determined peptide length and with two positions within the peptide of conserved occupancy, called anchor positions. The information obtained in this way is summarized as peptide motif [16]. Such motifs can also be determined by comparing many individual peptide ligands [28]. In the case of H-2Kd, a motif based on binding of synthetic peptides was found to be essentially identical to the basic motif based on natural ligands [36]. The basic motifs of some class I alleles are shown in Table 1. In addition to the basic motif information, i.e. allele-specific length, anchor position and occupancy, the different motifs have more subtle characteristics, such as preferential use of certain residues at certain positions; for example, HLA-A2 ligands have a preference for valine, isoleucine, leucine or alanine at position 6, and H-2Kb ligands prefer tyrosine at position 3 [16]. Such positions have been called auxiliary anchors. If one attempts to use the allele-specific peptide motifs for the prediction of natural T cell epitopes [37], as has already been done successfully [38•,39•], one should consider the more subtle preferences as well.

Table 1. Natural MHC ligands. Examples of basic allele-specific peptide motifs for class I molecules.

Class I allele	Position									Reference
	1	2	3	4	5	6	7	8	9	
H-2Kd	X	Y	X	X	X	X	X	X	L	[16]
									I	
H-2Kb	X	X	X	X	F	X	X	M		[16]
					Y			I		
								L		
H-2Db	X	X	X	X	N	X	X	X	I	[16]
									L	
									M	
HLA-A2	X	L	X	X	X	X	X	X	V	[16,29••]
HLA-B27	X	R	X	X	X	X	X	X	X*	[28]

The common features of peptide ligands of the individual MHC molecules are shown. X stands for any residue. Amino acids are represented by the one letter code in bold. The side chains of these amino acids contact the allele-specific pockets in the class I molecule. For example, Kb-ligands are nonamers, with anchors at positions 2 mainly for the aromatic residue Tyr and 9 for the aliphatic residues Leu and Ile. The more detailed characteristics of the individual motifs have been omitted for clarity.
* Predominant usage of Arg or Lys

The side chains of anchor amino acids as well as of auxiliary anchors are thought to be held by the allele-specific pockets of the MHC class I peptide binding groove. The remaining residues of the peptide ('pointing up') should then be available for contact with the TCR [9•,10•,11–18,40–46].

Natural MHC class I ligands

The first three natural MHC class I ligands were identified in 1990 [22,23]. All were of viral origin, and the approach used was to compare in T-cell assays the natural MHC ligand with synthetic peptides in the case of the H-$2D^b$ restricted vesicular stomatitis virus (VSV) peptide Arg-Gly-Tyr-Val-Tyr-Gln-Gly-Leu, the natural ligand, isolated by high performance liquid chromatography, was partially sequenced. In these instances, knowledge of the viral protein sequence from which the peptides were derived was mandatory for identification. These and similar experiments also showed that exact peptide length (of synthetic peptides to be tested) is important for optimal T-cell recognition, since adding or removing one residue from the natural peptide size can decrease T-cell recognition (and MHC binding) by several logs [33,34,47–49].

In 1991 the first natural MHC ligand was sequenced directly in the absence of information on the protein from which it originated [16]. It was found later that this peptide, the H-$2K^b$-ligand Ser-Tyr-Phe-Pro-Glu-Ile-Thr-His-Ile, is derived from the protein tyrosine kinase JAK1 [50] (A Wilks *et al.*, unpublished data). Since then, the number of natural MHC ligands that have been characterized is growing exponentially [2,4•,25–28,29••,30•,51•,59•,52,53]. Most ligands known to date are self peptides, i.e. peptides derived from normal cellular proteins, representing the physiological MHC class I ligands. These come from all kinds of proteins synthesized in the cell and include proteins like histones, heat shock proteins, enzymes and so on. Relatively few natural class I ligands derived from foreign proteins, e.g. from pathogens, have been identified since 1990 [39•,51•], although the allele specific peptide motifs combined with T-cell recognition data of synthetic peptides, and sometimes peptide binding experiments, allowed the prediction of a large number of candidates for natural ligands (reviewed in [52]). Some examples of endogenous, as well as foreign peptides, naturally found associated with class I or class II molecules are listed in Table 2.

A technical development greatly improving direct sequencing of MHC eluted peptides was the use of tandem mass spectroscopy [29••]. This technique is ideally suited to dissect the thousands of different peptides eluted from a given MHC molecule, to obtain sequence information on individual peptides of relatively high abundance and to roughly estimate peptide complexity. Still a technical challenge, however, is to directly identify the peptide antigen recognized by a given T cell, if the origin of the peptide is unknown, as is the case, for example, for minor histocompatibility or tumor-associated antigens (the former are peptides derived from normal self proteins that can be recognized by foreign T cells). One of the reasons is the relatively low abundance (100–500 copies per cell) of most natural class I ligands as recognized by T cells of immunological interest [33,39•,51•,53].

Processing of MHC class I ligands

It is not known where and how MHC class I ligands are processed. Most peptides appear to be degradation products of proteins, although some alternatives have been discussed [54–56]. Many proteins donating peptides to class I molecules are located in the cytosol, and proteins artificially loaded into the cytosol can yield class I ligands [57–59]. In addition, however, probably all proteins synthesized in a cell, including those in the mitochondria [60,61], can supply class I ligands. The following is a widely held view for class I restricted processing: proteasomes cut cytosolic proteins into peptides, and products of *Tap1* and *Tap2* genes transport these peptides across the endoplasmic reticulum membrane where they bind to MHC class I molecules. This model, however, is still speculative regarding the postulated function of TAP molecules [62–64] and fails to take into account the observed MHC-dependency of peptide occurrence in cells [21,22,24•,51•,65,66•,67,68]. (For example, the male specific H-Y peptide, recognized by H-$2D^b$ restricted T cells, is apparently absent from male cells not expressing H-$2D^b$) [21]. With regard to this aspect, and on account of the structural features of the consensus motifs, we

Table 2. Natural MHC ligands. Examples of natural ligands of MHC class I and class II molecules

MHC allele	Peptides*	Origin	Reference
H-$2K^d$	S Y I P I I T H I	Protein tyrosine kinase JAK1	[17,50]
H-$2K^b$	K G Y V Y I Q G L	VSV nucleoprotein	[23]
	S I I N F E K L	Ovalbumin	[39•]
HLA-A2	I L K M V D F Y I V	Unknown	[29••]
HLA-B27	K R Y Q K S I F L	Histone H1	[28]
	R R I K I I V K K	HSP 70kD	[28]
H-$2D^b$	S P S Y V Y H Q I	VSV nucleoprotein	[16•]
	S P S Y V Y H Q F	VSV virion protein	[16•]
H-$2A^d$	I I Q F I Q Q I R	Mouse apolipoprotein I	[16•]
	Q F I Q Q I R I Q	Mouse apolipoprotein I	[39•]
H-$2A^k$	D G S T D Y G I I	Hen egg white lysozyme	[69]
	D Y G I I Q I N S	Hen egg white lysozyme	[69]
HLA-DR1	L R K P P K P V S	Invariant chain	[39•]
	K M R M A I P I I	Invariant chain	[39•]
	A I I I I R A M A	Unknown	[69]

*Anchor residues in bold. Amino acids are represented by the one letter code. HSP, heat shock protein; VSV, vesicular stomatitis virus.

speculated that the MHC itself might have an instructive role in peptide processing, in that larger peptide precursors, cut by conserved endopeptidases (proteasomes?), would first bind to MHC molecules and then be trimmed to the final size specific for the particular MHC allele [16,21]. Trimming of a leader peptide, probably in the endoplasmic reticulum, is suggested by the presence of longer peptides in addition to the core nonamer bound to HLA-A2-molecules -[29••,30•,31•]. Our speculative model would also explain the fact that in all human tissues and mouse strains examined, cells are able to process the very same peptides for one particular class I molecule, H-2D^b [21,24•,65,66•,67,68]. If one assumes, according to the other, more popular, model that the final peptide is produced before its first contact with the MHC molecule, all cells in a white mouse, for example, should constantly produce all the tens of thousands of MHC class I ligands not only for all the black, brown and other members of the species but also for all other mammals [69]. In addition, this high number of potential ligands should have an extremely short half life, since so far they have not been detected, in spite of thorough experimental attempts [66•].

The pathway of class I restricted processing is certainly an area attracting much attention at present. Particularly interesting molecules are the *Tap 1* and *Tap 2* gene products, proteasomes, and other potential transacting elements [70]. TAPs are essential for proper antigen processing; their exact function, however, is not known. It is known that class I molecules are peptide transporters (from the endoplasmic reticulum to the cell surface) but it is not known whether TAPs also transport peptides.

Considerations on the role of MHC class I molecules

Peptide selection on the cell surface of peptides derived from cellular proteins provides the immune system with the means to control the cell's interior. For a given individual, this control covers only a small portion of the expressed genome. Each individual in an outbred population (man or mouse) expresses four to six different class I molecules. Each molecule species can present an estimated 1 000 different peptides on a cell [29•,33,69]; since the peptide specificities are different, depending on the class I allele, each molecule species presents a different set of peptides. The peptide selectivity of a given class I molecule is such that about 1 per cent of random (nona-) peptides fit to it [33]. Thus, only about 4–6 per cent of the expressed genomic sequences fit into class I molecules in an individual but due to MHC polymorphism, a much higher percentage of these sequences fit to the various class I molecules expressed in an entire species. During their ontogeny, T cells are made tolerant [71,72] to those self peptides that are actually presented by MHC molecules, but T cells are not tolerant to the remaining 95 per cent of self protein sequences [68,73,74]. Although not sufficient to control for every point mutation in the expressed genome, this system is sufficient to report the occurrence of new (non-self) gene products on the cell surface, since about one peptide (1 per cent) fitting into a particular class I molecule should be present within a protein sequence of 100 amino acids. Thus, T cells have a chance to detect cells expressing genes from invading viruses or other organisms with cytosolic parasitism. In addition, the system should allow the detection of proteins not usually expressed (and, therefore, not inducing tolerance) such as genes normally involved in embryonic development that are aberrantly expressed in malignant cells [75–79]. Normal proteins expressed at unusual abundancy might also be detectable by T cells, since they can detect quantitative differences in the peptide copy number presented by cells [51•]. Thus, the system of class I restricted peptide presentation and class I restricted T cells is able to efficiently control intracellular viral and certain other (cytosolic) parasites [39•,80–82], and should also have some control on malignancies [75–79].

Could the system be more efficient if the cell would present only the foreign peptides? Yes, indeed, but the cell cannot distinguish self from non-self; the immune system had to evolve a sophisticated system for this challenge, consisting of many cells, tissues, and organs, most notably the thymus and lymphocytes.

Class II

Structure of class II

It is believed that class II α and β domains form a groove that is similar to that formed by the the α1 and α2 domains of class I molecules [83,84]. This notion has been derived from modelling the class II sequence according to class I crystallographic data [4], since a photograph of a class II crystal has been taken [85•] but no X-ray crystallography has been reported yet. From the nature of natural peptide ligands of class II molecules reported since 1991 [86•,87,88•,89–92], it can be deduced that there must be certain differences in the peptide holding groove compared with that of class I molecules. The most notable difference is that the ends of the groove appear to be open, that is, to allow overhanging of both amino and carboxyl termini of bound peptides. The latter are probably in an extended conformation rather than in the form of α-helices.

Natural ligands of class II molecules

Our knowledge of class II physiology was more advanced than that of class I physiology for many years. T helper cells were first found to be MHC class II restricted in their interactions with B cells two years before MHC class I restriction of cytotoxic T cells was demonstrated in 1974. The first naturally processed MHC ligands were also eluted from class II molecules, although they were not identified [93]. Our understanding of class I molecules surpassed that of class II with the X-ray crystallographic study of class I structure in 1987 [4] and with the identification of natural ligands in 1990 [22,23].

The first natural MHC class II ligands to be identified were eluted from mouse H-2A^b and H-2E^b molecules [85•]. These peptides were between 12 and 18 amino acids

long the carboxyl terminus appeared to be 'ragged', i.e. peptides with a given core sequence could be extended at the carboxyl terminus to give 15-, 16-, 17-, or 18-mers. Again, the use of tandem mass spectrometry provided a boost for the identification of naturally processed class II ligands [88•]. Use of this technique allowed the detection of up to 2000 different ligands on a single class II species, H-2A^d, although only the 12 most abundant ones have been sequenced. To date, information is published on H-2A^b, -E^b, -A^d, -A^k, -A^k, and HLA-DR1 peptide ligands, representing some 40 natural peptides, some of which are indicated in Table 1 [86•,87,88•,89–92].

Both the amino and carboxyl termini are ragged; their length can vary between 12 and 25 residues, with the majority apparently around 15 amino acids. For T cell recognition, the length of the peptide seems to matter little, as long as a certain core is maintained [91,92].

Allele-specific peptide motifs?

The sequencing of the first 13 natural MHC class II ligands (from H-2A^b and H-2E^b molecules) did not indicate any obvious motifs [86•]. Subsequent studies on the 11 most abundant H-2A^d associated peptides indicated that peptide binding motifs established earlier using synthetic peptides [93,95] were contained within the natural ligands that associate with H-2A^d [88•]. Similarly, HLA-DR1 eluted peptides were aligned to conform to a motif of three anchor-like positions [90]; this motif, however, did not exactly conform to HLA-DR binding motifs established with synthetic peptides [89,96–102]. Common to all studies analyzing natural class II ligands is the ragged end of peptides; that is, peptides containing a given core sequence were found in various lengths, with extensions both at the amino and carboxyl termini. Thus, there appear to be allele-specific peptide motifs in class II ligands, although the motifs are not as obvious as with class I ligands, and the anchor-like positions may allow a broader spectrum than in the case of class I molecules. Although crystallography studies on class II molecules have not been reported yet, the information on class II restricted peptides suggests that allele-specific pockets will also be found in the groove, and that both ends of the groove are 'open' to allow overhanging of peptides at both sides, explaining the different length of peptide ligands. This is illustrated in Fig. 1.

Most class II ligands are of endogenous origin

It is a widely held misconcept that class II molecules predominantly present peptides from exogenous anti-

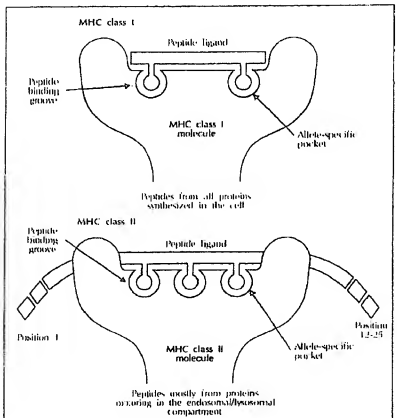

Fig. 1. A simplified view of MHC class I and II molecules as peptide receptors. Class I molecules hold peptides of eight to 11 amino acids with both amino and carboxyl termini tightly fixed in the groove. Two side chains or anchor residues of the peptide are protruding into complementary allele-specific pockets of the groove. Class II ligands, consisting of 12–25 residues, are probably also held in the groove by anchoring two or three peptide side chains into complementary structures of the class II groove. In contrast to class I, however, both peptide ends are not fixed in the groove but are allowed to 'hang out' of the end of the groove.

gens, i.e. from antigens taken up by the antigen-presenting cell. As can be seen from the list of known natural class II ligands [86•,87,88•,89–92], most are actually endogenous to the cell. Peptides are derived from other MHC or MHC-associated molecules, e.g. HLA-A2, I1-2A or -E, from the invariant chain, retroviral protein, or the transferrin receptor. In addition to these endogenous peptides, foreign peptides are also found, e.g. peptides derived from bovine serum albumin present in the cell culture medium used to propagate the cell line [86], or peptides derived from antigen deliberately 'fed' to the cells in high concentration, such as hen egg white lysozyme [92]. Common to most of the proteins donating peptides to class II molecules is their potential to occur in the endosomal/lysosomal compartment of the cell, no matter whether this is achieved by phagocytosis of exogenous proteins, or by directing endogenous molecules into this compartment.

Thus, class II molecules predominantly present peptides derived from proteins occurring in, or directed to, the endosomal/lysosomal compartment of the cell, whereas MHC class I molecules can present peptides derived from all the proteins synthesized in a cell.

How do the cells process the peptides?

MHC class II ligands are probably produced by lysosomal enzymes (reviewed in [103]). Several enzymes, among them cathepsin D, are involved, as indicated by the different sensitivity of particular ligands to protease inhibitors (reviewed in [104]). It is not known, however, whether the lysosomal enzymes produce the final size ligands before binding to class II molecules, or whether the ligand is trimmed after binding, with protection of the peptide core sliding in the groove ('determinant protection') [105]. The ragged ends of class II ligands may suggest limited action of exopeptidases on the ligands after binding.

Why are MHC class II molecules not flooded with ligands in the endoplasmic reticulum, where they are assembled, as are class I molecules? One reason for this is the blocking of the peptide binding site by the invariant chain. Only after removal of the latter in the endosomal/lysosomal compartment do the peptides have access to the class II cleft [106].

T cell function and MHC class II molecules

The function of class II restricted $CD4^+$ T cells is twofold: firstly, they 'help' other antigen-specific lymphocytes (B cells and other T cells) to differentiate and to become activated; and secondly they attack foreign antigen presented by MHC class II positive cells, either directly or by activation of non-specific cells like macrophages or granulocytes. Both functions are mediated and regulated by differential cytokine production [107]. For the interaction between T helper cells and B cells, the requirement for the T cells to recognize the class II ligand on B cells forces close contact between the T cell and the B cell recognizing the same antigen and allows directed delivery of cytokines. The epitopes recognized by the TCR, however, are usually not the same as the antibody epitopes.

In addition to positive immune responses, recognition by T cells of peptide ligands associated with MHC molecules may also lead to tolerance, not only in the thymus or during T-cell ontogeny. Resting B cells, for example, presenting antigen acquired by receptor mediated phagocytosis, have been hypothesized to induce anergy in mature T cells that recognize them [108,109]. This could be a mechanism for maintaining self tolerance in the mature T-cell compartment; the mechanism might also be used to induce peptide-specific tolerance in T cells artificially.

Concluding remarks

Comparing the peptide receptor characteristics of MHC class I versus MHC class II molecules, two major differences are evident (see also Fig. 1). First, the majority of peptide ligands of most class I molecules have a distinct length (eight or nine residues), depending on the allele, whereas MHC class II ligands may vary considerably, from 12–25 residues. The second difference is regarding the peptide motifs. Class I molecules have distinguished allele-specific motifs which become obvious on aligning as few as nine or 10 natural ligands, and are easily accessible by pool sequencing of the total ligand mixture. Peptide motifs of class II molecules are not as obvious, especially if one looks at a few ligands only. Nevertheless, class II specific peptide motifs appear to exist, and additional work is required to establish clear motifs for the different class II alleles.

Knowledge of such motifs, for class I and class II molecules, is useful to predict natural T-cell epitopes. Other subjects of current and future interest include: firstly, the peptide specificities of HLA-C [110] and of non-classical class I molecules (in addition to the Qa-2 motif [111] and the information on H-2M3, which is specific for N-formylated peptides [60,61]), and also peptide specificity of HLA-DQ and HLA-DP molecules; secondly, the fine dissection of the molecular interactions in the peptide–MHC–TCR relation (in particular, which atoms of the three molecules contact each other?); thirdly, the dissection of the processing pathways involved in processing of MHC ligands; and finally, the identification of peptides expressed on thymic epithelium and elucidation of their role in positive selection.

The detailed information of the peptide receptor function of MHC molecules, especially the structural features of their ligands, should be useful for applied immunology, including vaccine development, immunotherapy of malignant and infectious diseases, and prevention and therapy of autoimmune diseases.

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Due to constraints on the space allowed for this review much of the work that led to the model of peptide processing for MHC class I molecules has not been cited.

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The Discovery and Use of HLA-Associated Epitopes as Drugs

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ABSTRACT: MHC receptors "display" peptide fragments to T cells. These peptides are predominantly derived from proteins expressed within or ingested by the presenting cell. Since empty MHC molecules are highly unstable, peptide ligands are bound prior to MHC surface expression and the ensuing $t_{1/2}$ off rates are often on the order of days. It is the remarkable stability of MHC/peptide complexes, which provide us an opportunity to purify MHC molecules from infected, transfected, or antigen pulsed cells and subsequently identify the naturally processed peptides being presented. On the other hand, the stability of MHC/peptide complexes substantially reduces the potency of parenterally administered peptides *in vivo*. Using serial immuno-affinity chromatography and mass spectrometry, naturally processed peptides can be identified. When these peptides are then encoded into nucleic acid and delivered parenterally, they are highly immunogenic. Application of these techniques to induce vigorous CTL responses will be discussed.

KEY WORDS: peptides, antigens, vaccines, DNA, microsphere, cytotoxic lymphocyte.

I. THE NATURE OF HLA-PEPTIDE INTERACTIONS

Proteins encoded within the class I and class II regions of the major histocompatibility complex participate in immune recognition of pathogens by binding and transporting to the cell surface small immunogenic peptides.[1] Class I and class II genes are the most polymorphic gene families in the human genome. This led to the proposal that a significant survival advantage has been afforded by increasing the diversity and number of these receptors. This concept is also supported by the binding mechanics of these molecules. Unlike most receptor-ligand interactions where specificity is imperative, HLA molecules strive to accommodate as many different ligands as possible. Most often the peptides presented by HLA molecules are remnants of full-length proteins that have undergone partial proteolytic degradation.[2,3] As essentially random combinations of twenty different amino acids, these peptides represent a remarkably diverse collection of chemical entities or ligands. From a receptor/ligand point of view, it is difficult to imagine how a single receptor could bind to all these peptides and do so with high affinity when these ligands are so seemingly different. The answer to this puzzle is that HLA molecules do not focus their attention on the differences between peptides but rather on the features that all peptides have in common. The universal features that HLA molecules have evolved to recognize are the peptide bonds between each amino acid and in the case of class I receptors to the NH_2- and -COOH termini of peptides.[4,5] HLA molecules can bind peptides with low nanomolar affinities by employing numerous cooperative interactions, which together add up to the overall high affinity.

Although a given HLA molecule can bind a large number of different peptides, it cannot bind all peptides. This fact resulted in the selective pressure to increase the number of receptors expressed. But, if HLA molecules use common structural motifs to bind to peptides why are they incapable of binding all peptides? Most peptides

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in the size range bound by HLA molecules are too small to maintain any rigid tertiary structure. Our working model of the HLA-peptide interaction suggests that during the binding process, an initial interaction at only a few contacts captures the ligand while the non-bound portion is allowed to freely rotate at each peptide bond in an attempt to increase the number of contacts. This model is supported by observations of weak initial affinities, which then convert to higher affinity-stable complexes.[6,7] The end-state conformation minimizes potential steric hindrances and appropriately aligns the electrostatic interactions of each charged amino side chain with those elements present within the receptor's ligand binding groove. If during this process irreconcilable conflicts are encountered, the weak preliminary interactions, which initiated the binding process, will prove to be insufficient to maintain a stable conformation and the ligand will be released.

Notwithstanding the above, the atomic idiosyncrasies (derived from polymorphisms with the binding pocket) of HLA molecules will tend to favor certain types of amino acid side chains at particular positions of the ligand. Although these amino acids only moderately participate in the total binding energies between receptor and ligand, they may make significant contributions during the critical early interactions between HLA and ligand. Small contributions in binding energy during this time period will dramatically increase the likelihood that the remaining cooperative sets of interactions proceed.

Interestingly, class I and class II HLA molecules are incomplete with respect to the structural requirements needed to completely fold. Obligate chemical contacts are contained within the bound peptide ligand and as a result "empty" class I and class II HLA molecules are unstable and unfold rapidly. This is not the case for their close structural relative, the neonatal Fc Receptor.[8] This implies that acquiring the appropriate ligand before achieving the tertiary fold is important. When one considers the fundamental role class I and class II molecules play as the first step in the immune recognition event, tight control over ligand acquisition makes sense.

HLA-peptide complexes have significantly slower offrates than most structurally rigid receptors-ligand systems. The offrates for most HLA-peptide complexes are similar in duration to the half-life of nascent HLA molecules. From a practical standpoint these facts have significant implications. Because HLA molecules and their ligands are assembled within intracellular vesicular compartments and the $t_{1/2}$ are often on the order of days, sparingly few HLA complexes on the cell surface are "empty" or "open" for binding of exogenously encountered peptides. Thus, a peptide-based pharmacologic agent, when administered parenterally, has little chance of loading HLA molecules (further discussed below) and remains immunologically inactive outside the context of HLA presentation. Yet, because of these slow offrates, HLA molecules can be purified from cells, and an analysis can be performed on the repertoire of peptides being presented.

II. DISCOVERY OF IMMUNOLOGICALLY ACTIVE PEPTIDES

One approach for identifying minimal epitopes has been to synthesize partially overlapping peptides covering the entire amino acid sequence of the target protein and then screen each of these peptides for their ability to bind to HLA molecules or elicit an immune response. The primary obstacle in applying this technique is that a significant fraction of the high-affinity-binding synthetic peptides identified do not correspond to the naturally processed epitopes presented by the cell and thus are artifacts. A more practical obstacle is the expense of synthesizing overlapping peptides covering the entire length of the protein, especially in those instances where the target protein is of an extended length. Nevertheless, several immunogenic peptides were identified using this approach.[9-12]

As an alternative to screening peptide libraries, techniques have been developed to identify naturally processed peptides from the surface of cells grown in tissue culture or tumors removed from patients. The pioneering work of Buus et al. was the first to show that acid treatment of HLA molecules released low-molecular weight proteinaceous material.[13] Refinement of this technique applied to purified HLA-peptide complexes has resulted in a substantial amount of sequence information defining HLA-associated peptides.[2,14-25]

The majority of these applications utilize immunoaffinity chromatography to purify the HLA molecules from either cell lines or human tissue samples.[26] Originally, soft carbohydrate gels were used for the preparation of chromatographic packings because they were inexpensive, easily derivatized, of high porosity, and useful for laboratory scale preparative separations. In our laboratory this protocol was modernized to improve the protein yield, reduce the number of manipulations, and eliminate the exposure of HLA-peptide complexes to extensive dialysis.[27] By automating the purification system, the time required to obtain highly purified HLA-peptide complexes can be reduced from several days to a matter of hours. This reduction in time is important to maintain the integrity of the HLA-bound peptide repertoire. Although most of these complexes are quite stable, the receptor-ligand interaction is not covalent and peptides are continually being released over time, as described in the previous section. Hence, lower affinity and lower occupancy peptides are naturally more difficult to isolate and analyze. In some instances immunogenic peptides fall into this category.[22,28] Thus, by implementing a faster purification scheme, a more complete analysis of the entire bound peptide repertoire is achieved, leading to a better chance of isolating and identifying relevant immunogenic epitopes. However, speed is not the only advantage of an automated system. Along with the advantage of faster overall purification times come improvements in reproducibility afforded by this approach that are necessary to increase the sensitivity of this technology for the analysis of complex biological samples.

The automated system described above consists of tandem HPLC columns linked in series to achieve immunoaffinity separations of several HLA molecules from a single sample. In this approach, mAbs are directionally attached to high-strength, large throughpore perfusion sorbents that allow fast velocity flowrates (up to 20 mL/min) and also facilitate the cleaning/recycling of columns after protein/lipid fouling.[29] This system was designed with multiple high-pressure switching valves, which allow appropriate flow paths for automated column loading and serial elution of up to five individual mAb-specific immunoaffinity columns (R. M. Chicz, unpublished results). These modifications empower a single system to automatically purify up to five allotype specific HLA molecules from a single lysate preparation without manipulation of the effluents or reloading of collected fractions. Because this is a modular system, additional high-pressure switching valves can be added to increase the number of individual columns to be eluted. This system is capable of both complex immunoaffinity protein purification as well as sensitive analytical reversed-phase chromatography (RPC) peptide separations, contiguously.[27] The effluent from the RPC column is split and single microliter aliquots robotically deposited onto a matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF/MS) target plate, while the remaining material is transferred into bioassay plates or storage vials. Next, an acidic matrix is applied directly on the sample plate and the peptide complex is crystallized. Once complete, the peptide samples are ready for immediate automated mass analysis.

The next step is to catalog the repertoire of peptides that are presented. To achieve this, a mass spectrometer capable of high sensitivity analysis is required as the monitoring device. Four prominent features make MALDI-TOF/MS an especially attractive analytical tool for this analysis. First, MALDI-TOF/MS spectra tend to be less complicated than those collected using electrospray ionization mass spectrometry (ESI/MS) because the ionization process favors the formation of single (1+) ions rather than multiply charged ions (1+, 2+, 3+, etc.). This is an important consideration when comparing spectra of complicated samples. Second, this technique uses minimal amounts of sample, sub-femtomole amounts for mass analyses and femtomolar amounts for sequence analyses. Third, the mass accuracy and superior mass resolution afforded using this technique are not achievable using alternative mass spectrometry analyses. Finally, primary sequence information can be generated using two complementary modes of daughter ion fragmentation. The first two considerations described above are self evident, but the remaining points are subtle and will be expanded on below.

A reflectron time-of-flight mass spectrometer is capable of collecting mass spectra in several modes of operation. Peptide fractions are first

screened for complexity and relative abundance using the linear mode of analysis, which has a lower resolving power and mass accuracy, but a higher transmission efficiency for complex mixtures. These spectra provide an accurate catalog of individual peptides present and consequently a precise time of elution from the RPC column. Because each fraction from the primary RPC separation can contain hundreds of individual peptides, high resolution combined with mass accuracy is the only method that can reliably screen the fractions for complete peptide characterization. Thus, the next analysis is performed in the reflector mode which increases the resolution and mass accuracy of the spectra by increasing the flight time of the ionized species. For example, techniques with lower resolving power (i.e. ion trap or triple quadruple mass spectrometers with normal resolution of ~1000 to 2000 in the m/z = 1000 to 2000 range at femtomole sensitivity) have difficulty characterizing peptides with mass differences of 1 to 3 Da or less (Figure 1a). The difficulty is mostly due to the inability of these alternative techniques to properly resolve the isotopic distribution of a single peptide. MALDI-TOF/MS instruments equipped with extended flight paths and delayed extraction ionization fields

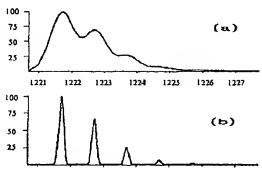

FIGURE 1. Isotopic resolution profiles for several different mass spectrometry techniques. The upper panel represents the theoretical isotopic resolution for a m/z = 1221.7 with a resolution of 1500. This is a typical value for electrospray ionization triple quadrupole mass spectrometers under limiting sample conditions. The lower panel represents the actual mass spectra collected in the high resolution reflector mode for the same peptide using MALDI-TOF/MS.

can achieve superior mass accuracy and resolution (~15,000) (Figure 1b),[30,31] at the femtomole and even attamole level. The exceptional performance of this instrumentation enables the reliable collection of multi-component spectra while permitting the mathematical subtraction of one spectra from another. Coupled with highly reproducible chromatographic separations, subtractive analysis of naturally processed peptides from antigen pulsed and non-pulsed cell lines can be performed. The application of this technology is utilized to identify novel HLA-associated peptides derived from immunogenic target proteins without the aid of T cell assays.

Another advantage of MALDI-TOF/MS relates to its ability to generate sequence information on peptide samples. Fragment ions can be generated in reflectron MALDI-TOF/MS by a phenomenon described as post-source decay (PSD).[32] Briefly, the sample analyte ions undergo "delayed" fragmentation/neutralization reactions during flight stemming from multiple collisions with matrix molecules during gas phase plume expansion and ion acceleration. It appears that MALDI-TOF/MS is unique in forming pre-excited precursor ions that move at a fairly high kinetic energy over a long distance where they can undergo uni-molecular decomposition with or without further collisional activation.[33] Using PSD analysis, complete sequence information can be generated from the daughter ion fragmentation patterns (Figure 2). The fragmentation patterns are different from those observed using high energy 4-sector instruments or other tandem mass spectrometers, such as electrospray triple quadrupole instruments. Furthermore, sensitivity is at least two orders of magnitude better than the aforementioned mass spectrometry approaches due to higher overall yield of fragment ions and higher ion transmission inherent in TOF instruments.[33] However, to enhance PSD analysis even further, a collision cell can be introduced to the system. With a collision cell in place, high energy collision-induced dissociation (CID) spectra can be collected, which produce complementary fragmentation patterns as compared to PSD spectra. The combined data sets produce additional structural information for the sequence determination of unknown peptides (Figure 3). Unfortunately, there are some practical limitations inherent with PSD

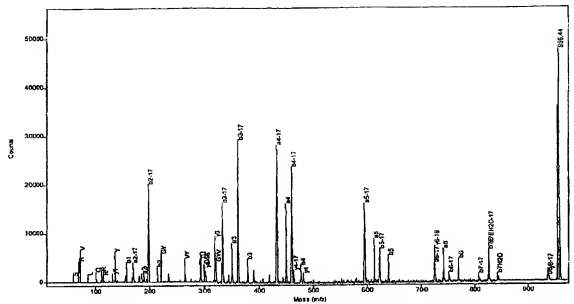

FIGURE 2. PSD fragmentation pattern from MALDI-TOF/MS analysis of a MHC-associated immunogenic peptide from vesicular stomatitis virus. A complete daughter ion fragmentation pattern is observed.

analyses. The current instrument design of commercial MALDI-TOF mass spectrometers is optimized for high resolution data collection, not sequence analysis. This characteristic of the instrument design is most evident when analyzing complex multi-component mixtures. Although the linear and reflector mode are capable of detecting most of the individual ions in a complex mixture, the suppression effects resulting from the plume ionization of a multi-component mixture are manifested by a decrease in sensitivity during PSD analysis. Innovations in ion gating and detector design are addressing this issue that should result in an increase in sensitivity for sequence analysis (Proceedings of the 45th ASMS Conference on Mass Spectrometry and Allied Topics, Palm Springs, CA, 1997). Unfortunately, the rapid progress of this technology still needs to await applications testing and marketing before commercial instruments are available.

A complementary technique to MALDI-TOF/MS for the sequence analysis of low femtomole amounts of peptide is ion-trap mass spectrometry. Ion-trap mass spectrometry was recognized as an emerging technology for the sequence analysis of HLA-associated peptides prior to the availability of commercial instruments.[27] In the interim, improved mass accuracy, resolution and sequencing capabilities were achieved on commercially available MALDI-TOF mass spectrometers. However, the combination of these two technologies may present the optimal manner by which peptide sequence identification can be efficiently obtained from complex samples. The rationale for this proposal is listed below. First, the mass range of ion-trap instruments has recently been extended to include linear mass calibration and ion fragmentation for peptides.[34,35] With these advances in place, several commercial ion-trap instruments are now available. Briefly, the strength of the ion-trap technology is the capability to isolate a given ion while ejecting all the nonselected ions from the instrument, hence the name ion-trap. This is accomplished through the use of non-linear multipole fields, advanced resonance frequency electronics, and optimized ring and endcap designs in the trap, which enhance the ion ejection speed and extend the useful mass range of the instrument. The end product is the ability to perform multiple fragmentation experiments on a given ion (known

as $MS^{(n)}$, which extends the amount of information collected from peptide sequencing. This is manifested by performing a ZoomScan or limited mass range scan on a known mass. In this mode, the instrument can operate at high sensitivity and resolution, but at the cost of scanning only a limited mass range. The price for this optimization is the inherent weakness of lower sensitivity and resolution of normal full scan spectra of the parent ions. The decreased sensitivity and resolution compromises the detection of most ions in complex mixtures. For these reasons, the combination of MALDI-TOF/MS with ion-trap MS may lead to faster sequence identification of HLA-associated peptides.

Mass spectra collected using reflector MALDI-TOF/MS analysis normally have a mass accuracy near 0.01%. This is sufficient for use in mass matching protocols, where theoretical mass values of peptides are compared with a linear sequence from a target protein.[36–38] Novel mass values obtained by the subtractive algorithm are used to search out all possible mass matches within the amino acid sequence of the target antigen. Posttranslational modifications can be taken into consideration during these analyses. Those prospective peptide masses matching potential strings within the target antigen (within a tolerance of 0.02% using monoisotopic mass values) are further analyzed. Mass matching is useful because it focuses the ensuing analysis on sequence verification as opposed to complete unknown sequence determination. Because the mass matching protocol described above matches the linear peptide sequence with the experimentally reported mass value, the fragmentation patterns, including all ion types (b, y, a, d, w series), immonium series, and deamidated and dehydrated forms can be mathematically predicted. Thus, peptide masses chosen by mass matching can be sequenced and

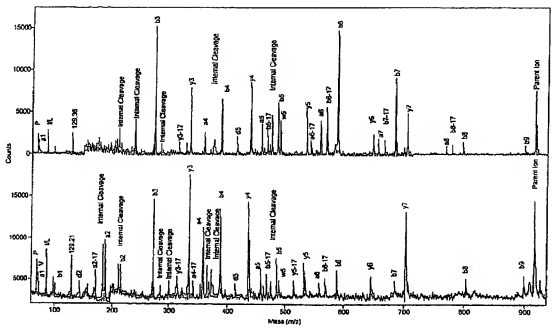

FIGURE 3. Comparison of PSD and CID fragmentation patterns using MALDI-TOF/MS. The upper panel represents the compiled PSD spectra (complete series of mirror ratio stitches) for an HLA-A2 specific peptide derived from an HPV viral protein. Notice the poor fragmentation series below mass 200. The lower panel represents the CID spectra (complete series of mirror ratio stitches) for the same peptide. The lower mass fragments are more pronounced using the CID method. Combining both sets of data promotes a more complete sequence analysis, thus making the determination of an unknown peptide more likely.

the experimentally determined PSD and CID spectra (collected by either MALDI-TOF/MS or ion-trap MS) are compared with the theoretical predicted spectra to verify the mass matching by sequence analysis. Once a candidate peptide is properly identified, synthetic peptide analogues are produced and HPLC retention, mass analyses, and most importantly PSD and CID fragmentation patterns are collected and compared with those used to originally determine the unknown sequence to confirm the unknown sample determination. After verification, the candidate sequence is tested in immunological systems.

III. PEPTIDES AS DRUGS

The ability to determine the precise fragments bound by HLA molecules is a remarkable advance in structural immunology. However, unless these advances can be translated into improved pharmacologic agents they will represent a mere technical feat without broad value. The traditional method for generating cellular immune responses against defined antigens has been to administer full-length proteins or synthetic peptides mixed with adjuvants. As the name implies, adjuvants are used to boost the immunogenicity of these antigens. In many instances these techniques were effective in generating humoral responses, but cellular responses have proven to be more difficult to activate. It has been generally postulated that by administering the precise peptide sequences recognized by T cells, the presentation efficiency and thus potency of these antigens would be increased. In most instances this has not turned out to be the case.[39] The primary obstacles are related to inefficient cell surface binding of the administered peptides and the need for prolonged stability *in vivo*.[40] Synthetic peptides are rapidly degraded by serum peptidases as well as cleared by normal hepatic and renal function.[41] One alternative to using conventional synthetic peptide mixed in adjuvants has been to use modified lipopeptides.[42] These peptides have improved serum stability and use the lipid component to increase cellular permeability. Lipopeptide formulations showed higher potency in animal systems when compared with the standard peptide approach, and human clinical studies are ongoing.[43]

IV. DNA-ENCODED ANTIGENS

Injection of plasmid DNAs encoding antigens into muscle or skin is becoming a well-studied approach to generating immune responses. In this system strong viral promoters are used to overexpress antigens. In some cases, deleterious effects may result from an overabundance of viral antigens and may lead to cell transformation.[44–47] Potential complications arising from this scenario should be avoided if DNA vaccines are to be safely used in humans. One alternative is to use only the naturally processed viral or tumor epitopes to activate T cells. Moreover, providing cells with the pre-processed antigenic peptide bypasses the processing machinery and is a more efficient method in loading HLA molecules with antigen.[48] In addition, by using small fragments as immunogens the potential complications or toxicities associated with the expression of functional proteins are minimized.

Protective immune responses were demonstrated using recombinant vaccinia constructs encoding CTL epitopes.[49–53] However, it has remained unclear whether the coincident immune response to vaccinia virus and the bystander cytokines produced have contributed to the immunogenicity of the antigenic peptides studied. Interestingly, certain palindromic sequences present in some bacterial plasmid DNA stimulate secretion of IL-2 and gamma interferon.[54–58] Cytokines induced in this way amplify T cell reactivity to protein antigens encoded in plasmids[55] and might also provide "help" for a response to preprocessed antigens. Plasmid DNA represents an immunization vehicle that is amenable to repeat injections, does not exempt patients with preexisting immunity to viral vectors, and does not pose risks associated with recombinant viruses.[59] Following intramuscular injection of plasmid DNAs encoding ER-targeted, naturally processed viral epitopes activate CTL responses. We studied this approach in several viral systems, and an example of two are shown in Figure 4. In each model studied, CTL activation was demonstrated; however, to achieve robust activation, this approach requires substantial amounts of plasmid DNA and repeated immunizations. These requirements were surprising because after the first injection muscles expressed

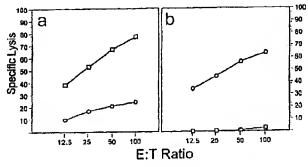

FIGURE 4. Induction of CTL activity following immunization with plasmid DNAs expressing minimal CTL epitopes. Mice were immunized with plasmid DNA encoding leader peptide tagged to amino acids 325 to 332 from the N protein of Sendai virus (panel a) or amino acids 52 to 59 from the N protein of vesicular stomatitis virus (panel b). Animals receiving the SV construct were immunized three times whereas the animals immunized with the VSV construct were only immunized twice. All booster immunizations were performed on 21 d schedules. CTL activity was measured on syngenic targets labeled with either the SV peptide (□) or the VSV (○).

the encoded antigens for extended periods of time (data not shown).

V. FORMULATIONS TO GET DNA INTO CELLS

The primary difficulty in using peptide-based antigens is achieving sufficient binding of HLA molecules. The primary obstacle for nucleic acid-based antigens is getting the DNA inside the cells and, perhaps even more importantly, getting it inside the correct cells. As demonstrated above and in numerous other systems, intramuscular immunization with naked DNA results in activation of immune responses.[59] Until recently, the mechanism by which this occurs was unclear. The theoretical problem has focused on how transfected muscle cells could activate naive T cells given the fact that they do not express co-stimulatory molecules required for initiation of a T cell response. Recent work suggests that the immune response is not initiated by transfected muscle cells but rather by bone marrow derived cells presenting antigen secreted or released from the muscle cells.[60,61]

Another approach was developed in which DNA is coated onto small gold beads which are then introduced through skin by a high pressure ballistic device.[63] This so-called "gene gun" is thought to function by introducing DNA into Langerhans cells that are known to professional antigen presenting cells. This technique requires substantially less DNA than the "naked" DNA approach but does require access to the moderately complicated ballistic device, and it does not disperse DNA systemically which may be desirable in certain therapeutic situations.

More recently an approach was developed to target DNA into professional antigen presenting cells. In this system plasmid DNA is encapsulated into particles composed of polylactide-co-glycolide (PLGA). PLGA microspheres are safe and reliable drug delivery vehicles.[62] Microparticles under ten microns are engulfed by phagocytic cells of the reticuloendothelial system which concentrates encapsulated material within professional APCs.[63] The propensity of polymeric spheres toward APC uptake makes them suited for intracellular delivery of DNA-encoded antigens. These antigens are synthesized by the cell and are accessible to the antigen processing machinery that loads HLA molecules and should accordingly activate T cells.

This new technique results in spheres with a median diameter of 3 to 5 μm. All steps of the procedure have been optimized to reduce DNA shearing and nicking and maintain the supercoiled nature of plasmid DNA. Over 80% is internal as determined by DNAase resistance. To demonstrate that these particles can be ingested and the DNA is released and expressed, a plasmid containing a luciferse cDNA was encapsulated and added to a culture of P388D1 (a murine macrophage cell line). Plasmid DNA expression as measured by luciferase activity is detectable at 24 h post particle ingestion (Table 1). Expression levels continue to increase for 3 d. After 5 d expression begins to decrease, due to cell death from overcrowding under these *in vitro* conditions.

The ability of professional APCs to ingest and express plasmid DNAs contained within microspheres implies that these kinds of vehicles could be used to elicit immune responses *in vivo*.

TABLE 1
Microparticles containing:

	Luciferase DNA	Control DNA
Day 1	1257	103
Day 2	2632	107
Day 3	3400	80
Day 5	763	90

Note: Expression of luciferase in P388D1 cells.

To explore this possibility CTL responses obtained by immunization with encapsulated and unencapsulated plasmid DNAs was compared. An example of these efforts are shown in Table 2. In this particular experiment, mice immunized either one time intraperitoneally with microspheres containing 2 to 5 μg plasmid DNA or immunized twice intramuscularly with a total of 200 μg unencapsulated, naked DNA. These data suggest that administration of encapsulated DNA is more potent than naked DNA, at least when CTL epitopes are used. One of the limitations of using naked DNA clinically is the amount of DNA needed. In the experiments described here, a single injection of microparticles containing ~2 to 5 μg DNA generates stronger responses than two immunizations with unencapsulated DNA using 100 μg in each injection. The increase in efficiency may be due to the protective nature of the polymer coating, but is more likely to result from the increased uptake of DNA by professional APC that are required for activation of naive T cells. Other studies demonstrated that significant immune responses can be generated following injection of DNA loaded microparticles into various routes. Furthermore, synthesis of targeted peptides within cells appears to overcome the limitations of synthetic peptides with respect to T cell activation. In fact, a recent report demonstrates that cells harboring a peptide-expressing DNA construct can produce >50,000 copies of HLA-peptide complexes on the cell surface.[44]

So it would appear that genetic immunization with a minimal amount of information from a viral antigen (i.e., the sequence of a naturally processed peptide) can be used to elicit significant CTL. Furthermore, encapsulation of plasmid DNA into PLGA microparticles increases the potency of DNA delivery over that seen with naked DNA or synthetic peptide and represents a simple and targeted mechanism for reaching antigen presenting cells of the immune system. As it is probable that immunological memory is dependent on residual antigen in lymphoid centers, this type of antigen delivery should ensure a prolonged memory response.[8] The long-term immunological memory to these nucleic acid-based antigens delivered in biocompatible polymers is ongoing.

VI. CONCLUSIONS

The presentation and recognition of antigen has recently been studied at the atomic level. These advances helped to clarify many of the problems encountered in our attempts to regulate the immune recognition. Although much is yet to be learned about the fidelity of T cell receptors, we are well on our way in developing systems to identify and deliver smarter antigens.

TABLE 2

Preparation		% lysis of target cells[a]
Naked BIOTOPE$_{vsv}$	200 μg	14.2 +/– 3.6[b]
BIOTOPE$_{vsv}$ ENSPHERE	2–5 μg	26.7 +/– 3.5[b]

[a] Data is reported as the mean lysis values from three individual measurements at an E:T of 50:1.
[b] Error is reported as the standard deviation; p value < 0.05 as determined by the Students t-test.
Note: Lysis values measured without *in vitro* priming to avoid introducing bias.

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